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1. Sugiyama et al., JOURNAL OF CLINICAL LABORATORY ANALYSIS (1994), 8(6), 437-42.

2. Sugiyama et al., ANALYTICAL BIOCHEMISTRY (1994) 218 (1), 20-25.

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An Enzymatic Fluorometric Assay for Adenosine 3′:5′-Monophosphate

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An enzymatic assay for adenosine 3′:5′-monophosphate (cAMP) is described. Current measurement techniques can be expensive, time-consuming, and lack versatility. The critical step of this new method is the enzymatic destruction of endogenous purinergic noncyclic nucleotides. The diester linkage of cAMP is then cleaved and AMP is phosphorylated to ATP. Newly formed ATP is amplified using ATP–ADP cycling reactions and NADPH is measured fluorometrically. cAMP was measured in neonatal rat ventricular myocytes cultured on standard 100-mm dishes and treated with 2 μM 3-isobutyl-1-methylxanthine ± 1 μM isoproterenol. When the enzymatic fluorometric assay was compared with an immunocolorimetric assay and a radioimmunoassay, cAMP content (pmol/plate mean ± SE) was 124.3 ± 6.7, 130.6 ± 3.9, and 144.0 ± 4.4 without isoproterenol and 656.4 ± 23.5, 659.5 ± 54.1, and 677.1 ± 48.9 with isoproterenol, respectively. The standard curve with the enzymatic fluorometric assay is linear, in contrast to the curves of the nonlinear immunocolorimetric assay and radioimmunoassay. The enzymatic fluorometric assay can be used to detect <20 fmol of cAMP/sample and can be adapted to measure <1 fmol/sample. It can also be used to measure the activities of adenylate cyclase and phosphodiesterase. In summary, this enzymatic cAMP assay is sensitive, safe, versatile, and inexpensive and has multiple potential applications.

Measurement of adenosine 3′:5′-monophosphate (cAMP) is essential to understanding fundamental mechanisms underlying signal transduction processes in nearly all living animal cells. While the currently available radioactive and nonradioactive immunoassays and high-performance liquid chromatographic methods are sensitive, they may utilize radioactive substrates and are often expensive and time consuming and lack versatility. Breckenridge initially described an enzymatic fluorometric cAMP assay for brain extract in 1964 (1). Although attempts have been made to improve this assay, the utility of the fluorometric enzyme-linked approach for measurement of cAMP in other organs such as heart and liver has been limited by extremely low tissue levels of cAMP and interference from endogenous adenosine-containing nucleotides that may be several orders of magnitude higher in concentration than cAMP (2).

To overcome these problems we developed a new method for measuring cAMP in which the initial reactions are designed to destroy all endogenous noncyclic adenine nucleotides and endogenous glucose 6-phosphate. This is a critical new step and is a significant improvement over previous fluorometric assays (1–3). In this report, we describe a highly sensitive cAMP fluorometric assay and its application to cultured rat ventricular myocytes. These measurements are compared to those from studies using a commercially available antibody-linked colorimetric assay and a radioimmunoassay. In addition, the potential broader applications of this new approach are discussed.

MATERIALS AND METHODS

Ventricular Myocyte Preparation

Isolated ventricular myocytes were generously provided by Dr. Joel Karliner and Mr. Norm Honbo from the University of California, San Francisco. Myocytes were prepared as previously described (4–6). Briefly, cells were obtained from hearts of 1-day-old rats, grown in primary cultures, and harvested by brief alternating cycles of room temperature trypsinization and mechanical dissociation. There were approximately 5 million viable myocardial cells per heart. After plating at a den-

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sity of approximately 1 million cells per 100-mm dish, cells were grown in minimum essential medium with Hanks' balanced salt solution containing 5% bovine calf serum cells (4,5). On Day 4, the medium was changed. The cultures contained >90% myocardial cells and cell numbers were constant over time (4–6).

In these studies, 6 plates of myocytes were randomized into two groups (n = 3 plates/group). The phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) was added to the medium of each group to achieve a 2 μM final concentration. After 5 min, isoproterenol was added to the stimulated group to achieve a final 1 μM concentration, while the control group received no additional drug. After 5 min, cells from all 6 plates and plates of culture medium without cells were eluted with a total of 5 ml of 100% ethanol. One-tenth of the eluent (0.5 ml) from each dish was removed and air-dried in a 12 × 75-mm borosilicate glass tube and stored at −80°C. The other 4.5 ml was similarly air-dried and stored. At the time of assay, the pellet was thawed and resuspended in 100 μl of 0.5 N perchloric acid. The extracts were vortexed at 4°C for 2 min and sonicated for 1 min using a sonicator (Branson Cleaning Equipment Co., A Smith–Kline Co., U.S.A.). The extract was neutralized with 25 μl of 2 N KOH and centrifuged at 2000g for 30 min, and 80 μl of supernatant was removed for assay.

Enzymatic Fluorometric Assay

This five-step assay utilizes the following series of reactions:

Step 1:

$$\text{ATP} \xrightarrow{\text{apyrase}} \text{AMP} + 2 \text{ phosphate}$$

$$\text{AMP} + \text{H}_2\text{O} \xrightarrow{\text{5'nucleotidase}} \text{adenosine} + \text{phosphate}$$

$$\text{Adenosine} + \text{H}_2\text{O} \xrightarrow{\text{adenosine deaminase}} \text{inosine} + \text{NH}_3$$

$$\text{Glucose 6-phosphate} \xrightarrow{\text{alkaline phosphatase}} \text{glucose} + \text{phosphate}$$

A volume of 3 μl of neutralized myocyte extract (either 2.16 or 0.24% of total eluent from a 100-mm plate) or 3 μl of a known amount of cAMP standard was added into a 10 × 75-mm Pyrex (Corning Laboratory Science Co.) assay tube. While at room temperature, 25 μl of "cleaning" reaction mix (100 mM Tris–HCl, pH 8.0; 2 mM $MgCl_2$; 2 units/ml apyrase; 2.5 units/ml 5′ nucleotidase; 0.1 mg/ml adenosine deaminase; 20 units/ml alkaline phosphatase) was added to each assay tube. The mixture was incubated at 37°C for 30 min. Enzymes were then destroyed by heating for 10 min at 90°C. A set of duplicate samples was similarly assayed and used for an internal tissue blank control (see below).

Step 2:

$$\text{cAMP} \xrightarrow{\text{phosphodiesterase}} \text{AMP}$$

A volume of 25 μl of a phosphodiesterase-containing reaction mix (25 mM Tris–HCl, pH 8.0; 2 mM $MgCl_2$; 0.02% bovine serum albumin; 40 μg/ml phosphodiesterase) was added to each assay tube from Step 1. A tissue blank was generated by adding buffer (25 mM Tris–HCl, pH 8.0; 2 mM $MgCl_2$; 0.02% bovine serum albumin) without phosphodiesterase to the parallel set of samples from Step 1. After 30 min at 37°C, the assay tubes were heated at 90°C for 5 min.

Step 3:

$$\text{AMP} + \text{ATP(trace)} \xrightarrow{\text{myokinase}} 2 \text{ ADP}$$

$$\text{ADP} + \text{phosphoenolpyruvate} \xrightarrow{\text{pyruvate kinase}} \text{ATP} + \text{pyruvate}$$

A volume of 25 μl of myokinase reaction mix (25 mM Tris–HCl, pH 8.0; 2 mM $MgCl_2$; 225 mM KCl; 20 nM ATP; 8 mM phosphoenolpyruvate; 2 mM dithiothreitol; 0.01% bovine serum albumin; 24 μg/ml myokinase; 50 μg/ml pyruvate kinase) was added to the assay tubes from Step 2. The reactions were incubated for at least 5 h and preferably and more conveniently overnight at room temperature.

Step 4:

$$\text{ATP} + \text{fructose} \xrightarrow{\text{hexokinase}} \text{fructose 6-phosphate} + \text{ADP}$$

$$\text{ADP} + \text{phosphoenolpyruvate} \xrightarrow{\text{pyruvate kinase}} \text{ATP} + \text{pyruvate}$$

A volume of 25 μl of hexokinase reaction mix (50 mM Tris–HCl, pH 8.0; 2 mM $MgCl_2$; 8 mM fructose; 0.01% bovine serum albumin; 350 μg/ml pyruvate kinase; 400 μg/ml hexokinase) was added to the assay tubes from Step 3. In view of the high concentrations of enzymes per reaction, it was important to set up these reactions at 0°C to ensure the same starting time for all assay tubes. After a 3-h incubation at 37°C, the reactions were terminated by heating at 90°C for 5 min.

Step 5:

$$\text{Fructose 6-phosphate} \xrightarrow{\text{phosphoglucoisomerase}} \text{glucose 6-phosphate}$$

Glucose 6-phosphate

$$+ \text{NADP}^+ \xrightarrow{\text{glucose-6-phosphate dehydrogenase}} \text{6-phosphogluconolactone} + \text{NADPH} + \text{H}^+$$

A volume of 800 μl of indicator reaction mix (50 mM Tris–HCl, pH 8.0; 100 μM $NADP^+$; 2 μg/ml phosphoglucoisomerase; 1 μg/ml glucose-6-phosphate dehydrogenase) was added to the assay tubes from Step 4. After 15 min at room temperature, the final concentration of NADPH was measured using a fluorometer (Optical Technology Devices, Inc., New York). The fluorometer was set such that a reading of 10 fluorometric units was equivalent to 1 nmol of NADPH in 900 μl of buffer (50 mM Tris–HCl, pH 8.0). Individual assay steps were often verified by concurrently assaying internal controls using known concentrations of the appropriate substrate (for example, additional ATP was used to check the cleaning reactions, an AMP standard curve was used to assess Step 3, and an ATP standard curve was used to assess Step 4).

Immunocolorimetric Assay and Radioimmunoassay

cAMP from the same myocyte extracts was also measured by commercially available cAMP immunocolorimetric assay kits (Amersham International plc, Amersham, UK) and radioimmunoassay kits (Amersham International). All samples were processed using the acetylated and nonacetylated methods for immunocolorimetric assay and the nonacetylated method for radioimmunoassay.

Biochemicals and Statistics

The enzymes and substrates used in this study were obtained from Boehringer-Mannheim Co., except for apyrase, 5′ nucleotidase, phosphoenolpyruvate, and fructose, which were obtained from Sigma Chemical Co. Measurements of cAMP using the enzymatic fluorometric assay, a colorimetric assay, and a radioimmunoassay were performed in triplicate. In some cases assays were performed up to six times/sample to assess assay variability. The results from each plate were averaged and reported as pmol cAMP/100-mm plate. The data are presented as means ± SE.

RESULTS

The cAMP content of cultured neonatal heart ventricular myocytes was measured using an enzymatic fluorometric assay. A representative cAMP standard curve is shown in Fig. 1. The standard curve has a slope of 47.2 and fluorescence readings from 36 to 144 over a cAMP concentration range from 0 to 2.24 pmol. The sensitivity of the cAMP assay is dependent upon a number of critical factors, including the amount of ATP added in Step 3, the duration of enzymatic cycling in Step 4, and the total assay volumes. In general, reduction in assay volume/tube will increase the lower limits of detection of cAMP. In these studies, a volume of 25 μl for each reaction was used because it is convenient to measure with an automatic pipette. When cAMP is assayed in the range 4–40 pmol, assay sensitivity can be decreased by using a 1-h cycling time and a 10-fold lower concentration of hexokinase and pyruvate kinase in reaction Step 4. In this specific concentration range, a higher concentration of ATP (4 pmol in each reaction tube) can be used in Step 3 and the time needed to complete Step 3 can be reduced to 2 h. Thus, the less sensitive form of this assay (>4 pmol cAMP/sample) can be conveniently performed in 6 h.

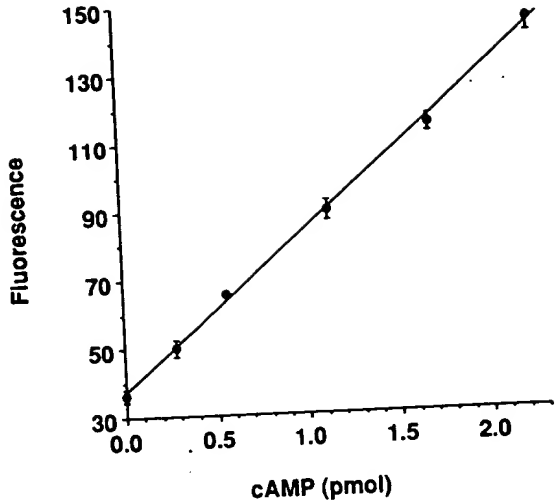

FIG. 1. A representative cAMP standard curve using the enzyme-linked fluorometric assay. The equation for the cAMP standard curves is $y = 47.2x + 37.0$. Ten fluorescence units are equivalent to 1 nmol/tube of NADPH. Data are expressed as means ± SE.

A 3-h cycling time was used in Step 4 to increase assay sensitivity. With 3 h in this step, differences as small as 20 fmol can be detected between different samples using the lower limit of detection, defined as 1 fluorescence unit divided by the slope of the cAMP standard curve (1 fluorescence unit = fluorescence of 100 pmol of NADPH with an excitation wavelength of 360 nm and an emission maximum at 460 nm) (7). Assay sensitivity can be further increased by altering the sensitivity of the fluorometer and/or extending the cycling reaction duration in Step 4.

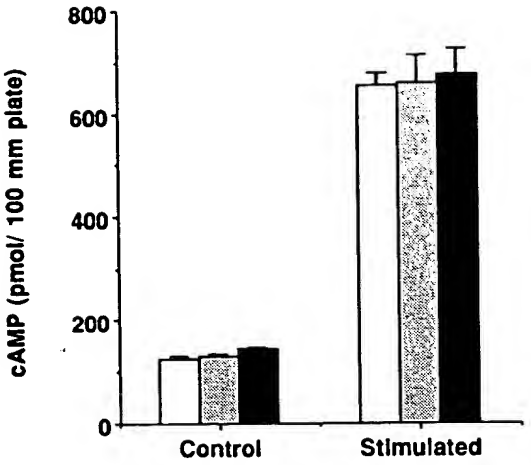

FIG. 2. Measurement of cAMP in neonatal cardiac myocytes using an enzymatic fluorometric assay (open rectangles), a commercially available antibody-linked colorimetric assay (shadowed rectangles), and radioimmunoassay (closed rectangles). Measurement of cAMP using these methods was performed with the same samples. The samples represent 0.24% of the total amount of cAMP extracted from a 100-mm culture dish (approximately 1 million cells/dish). Basal activities were measured in the presence of 2 μM IBMX and stimulated activities were measured in the presence of both 2 μM IBMX and 1 μM isoproterenol. Data are expressed as means ± SE.

The measurements of cAMP in control and isoproterenol-stimulated cells using this new fluorometric assay, a commercially available acetylated immunocolorimetric assay, and a nonacetylated radioimmunoassay are shown in Fig. 2. The measured values were similar with these three techniques. There was a fivefold increase in cAMP content in the cardiac myocytes after β-adrenergic stimulation. The data shown in Fig. 2 were generated from measurements of 1/10 of the eluent from each plate of cells. The measurements of cAMP in the more concentrated eluent (9/10 of the eluent from each plate) were ninefold higher and similar with all three assay techniques. For these methods of cAMP measurement, results of multiple analyses of a single sample did not differ by more than 10% with variability usually less than 5%.

It is known that tissue extracts prepared as described above may have intrinsic fluorescence and may contribute to the total fluorescence blank (7). In these studies the tissue blank values were essentially zero. Nonetheless, we routinely assayed extracts in the presence and absence of phosphodiesterase in Step 2 to ensure specificity of the assay. In the absence of myocytes, no cAMP could be detected in the medium.

Standard curves for cAMP using the acetylated and nonacetylated immunocolorimetric assays are shown in Fig. 3A, and that of a radioimmunoassay is shown in Fig. 3B. The nonacetylated immunocolorimetric assay standard curve, no longer recommended for use by the manufacturer, was not sensitive enough for measurement of cAMP in cardiac myocyte extracts. As these nonlinear curves are sensitive over a relatively small concentration range, careful measurement of more concentrated samples containing cAMP was critical in order to ensure that measured values were within this linear range of the standard curve.

The actual cost for the measurement of 100 samples of cAMP with this new fluorometric method was less than $30, at least 10 times less than the cost of the colorimetric assay kit or the radioimmunoassay kit. The total incubation time for the fluorometric cAMP assay varied between 4.5 and 9.5 h depending on its sensitivity, versus 4 h for the colorimetric assay and radioimmunoassay. Total working time with the fluorometric assay including the sample reading procedure varied between 5.75 and 10.75 h depending on its sensitivity, versus 6.25 h for the colorimetric assay and 6.83 h for the radioimmunoassay, respectively. However, both the fluorometric assay and the radioimmunoassay are conveniently performed using an overnight incubation step.

DISCUSSION

Given the biological importance of cAMP and the expense, time, radioactivity, and limited flexibility associated with the currently available assay techniques, we developed a highly sensitive, nonradioactive, enzyme-linked fluorometric assay to simplify the measurement of cAMP. Assay sensitivity can be easily varied over a broad range of cAMP concentrations, depending upon requirements of a particular experiment. This new approach can be used to detect changes of less than 20 fmol cAMP/sample. We applied this method to the measurement of cAMP in cultured cardiac myocytes. The concentration of cAMP was similar to levels reported by others (5,6). In addition, we compared the fluorometric measurement of cAMP to commercially available immunocolorimetric assay and radioimmunoassay, and the results were similar.

Previously we described a fluorometric assay for the measurement of adenylate cyclase activity in cardiac tissue (3). In those studies, stimulation of glycogen phosphorylase *a* by AMP was used to amplify the cAMP signal. Although the cleaning reaction steps of those studies and the current report are similar in principle, we added alkaline phosphatase in this present study to destroy glucose 6-phosphate and other potential interfering substances. In addition, we utilized a series of ATP–ADP enzyme-cycling reactions to amplify the cAMP signal in the present method rather than AMP-stimulation of glycogen phosphorylase *a*. Use of the ATP–ADP enzyme-cycling amplification step can lengthen the assay duration, depending upon the desired assay sensitivity, but it significantly increases assay sensitivity. Furthermore, due to the potential vari-

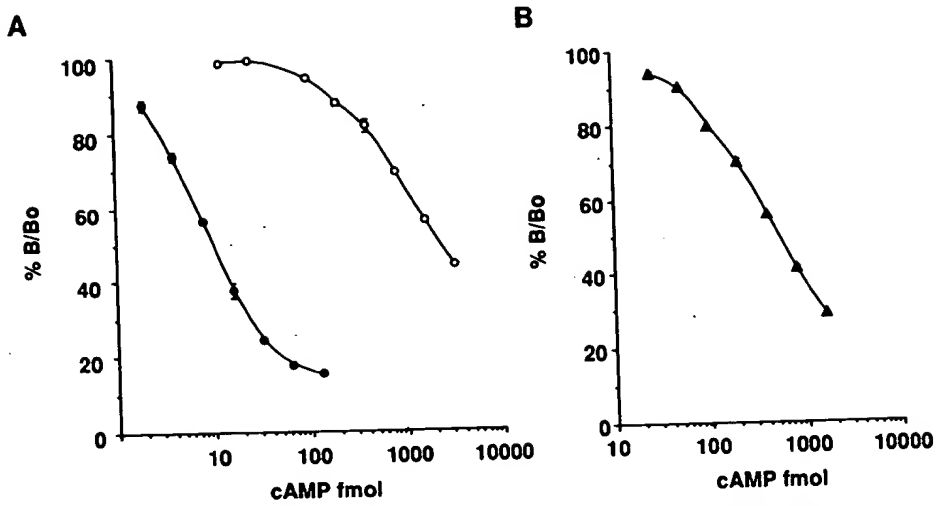

FIG. 3. (A) Representative cAMP standard curves using a commercially available antibody-linked acetylated (closed circles) and nonacetylated (open circles) colorimetric assay. (B) Representative cAMP standard curve using a commercially available nonacetylated radioimmunoassay. Data are shown as means ± SE. Both standard curves are generated by plotting the percentage B/B_0 as a function of the log cAMP concentration as follows. Colorimetric assay: $\%B/B_0$ = {(standard OD-NSB OD)/(B_0 OD-NSB OD)} × 100. Radioimmunoassay: $\%B/B_0$ = (standard or sample cpm/B_0 cpm) × 100. OD, optical density; NSB, nonspecific binding; B_0, blank; cpm, count per minute.

ability from lot to lot of glycogen phosphorylase *a*, at least in the currently commercially available forms (Sigma Chemical Co. or Boehringer-Mannheim Co.), the use of the ATP–ADP enzyme-cycling amplification step may be more reliable for measuring less than 1 pmol of cAMP/sample.

In these studies we arbitrarily adjusted the fluorometer so that a single fluorescence unit was equivalent to 100 pmol of NADPH. With settings adjusted in this manner, the sensitivity of the assay was calculated by dividing 1 fluorescence unit by the slope of the cAMP standard curve. As seen in Fig. 1, the limit of cAMP detection was 20 fmol with 3 h cycling. By adjusting the amount of ATP in Step 3, the ATP–ADP enzymatic-cycling times, the assay volumes in Step 4, and/or the fluorometer sensitivity, the assay can be used to detect less than 1 fmol cAMP. The reaction volumes, cycling duration, and concentration of enzymes, substrates, and cofactors described under Materials and Methods in this report provide convenient guidelines for measuring cAMP over a broad range (20–40,000 fmol).

Measurement of cAMP from the same cardiac myocytes extracts was performed with our new enzyme-linked fluorometric assay and a commercially available antibody-linked colorimetric assay and radioimmunoassay to compare and confirm our measurements with a currently acceptable cAMP assay kit. The measured values were similar in these three different techniques. However, the fluorometric approach may offer a number of potential advantages in that the standard curve is linear and can be used over a broad concentration range. Although a standard curve for a more sensitive form of the enzymatic fluorometric assay is shown in Fig. 1, the cAMP standard curve that can be constructed with this new approach is linear to greater than 0.1 μmol/sample. In addition, with the currently available immunocolorimetric assay used in the present studies, the nonacetylated less sensitive form of the assay cannot be used, while the concentration range in which reliable measurements can be made with the acetylated assay is quite narrow. Furthermore, the fluorometric assay is about 1/10 the expense of the other two assay kits on a cost/assay basis. It is noteworthy that the enzymatic fluorometric cAMP assay can be performed in microtiter plates, in a fashion analogous to the immunocolorimetric assays, as long as the final concentrations of NADPH are greater than 1 nmol/assay tube.

We elected to use the ATP–ADP cycling amplification method in these studies because it is rapid and reliable and high-quality enzymes are commercially available at minimal expense (1,7). Other alternative amplification schemes, in addition to the glycogen phosphorylase *a* method we have previously described (3), are also feasible. For example, it is also possible to amplify the ATP signal in Step 4 with a chemoluminescence assay for ATP that utilizes the luciferase reaction and firefly luciferin (8).

In addition to measuring cAMP in cultured myocytes, this assay can be readily adapted to measure cAMP in a wide variety of tissues. The same tissue extraction procedure can be used on frozen specimens (7). The new method is sensitive enough to measure cAMP in small biopsy samples weighing less than 0.1 mg from a number of different organs, such as heart, liver, and kidney, and can be adapted to measure less than 1 fmol cAMP/sample. Furthermore, it can be used to measure adenyl-

ate cyclase activity in a manner similar to the assay we have previously described (3) as well as phosphodiesterase activity by measuring the rate of cAMP degradation.

In summary, the nonradioactive, enzyme-linked fluorometric assay for cAMP offers an alternative to current techniques which are often more costly and less versatile. The example provided in this report demonstrates the potential widespread application of this new technique.

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Enzymatic Fluorometric Assay for Tissu cAMP

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cAMP is commonly measured using either immunoassay or high-performance liquid chromatography. The current methods are sensitive but may lack versatility and be expensive; also, radioactivity is potentially harmful to the operator and environment. Given these concerns, we developed a highly sensitive enzymatic fluorometric assay for cAMP. The method consists of five steps: (1) destruction of interfering compounds with apyrase, 5′ nucleotidase, adenosine deaminase, and alkaline phosphatase; (2) conversion of cAMP to AMP; (3) conversion of AMP to ATP; (4) amplification of ATP by ATP–ADP cycling; and (5) fluorometric measurement of resultant NADPH. cAMP was measured in male Sprague Dawley rats anesthetized with pentobarbital. Stimulated rats (n=4) received isoproterenol (16 μg/kg, s.q.) and aminophylline (20 mg/kg, s.q.), whereas controls (n=4) received no additional drug. With the enzymatic fluorometric assay, cAMP content in heart, liver, and kidney (pmol/mg wet wt, mean ± SEM) was 0.34 ± 0.03, 0.33 ± 0.03, and 0.92 ± 0.11 in the control group and 0.77 ± 0.10, 0.66 ± 0.04, and 1.53 ± 0.12 in the stimulated group, respectively. The total assay duration including sample reading procedure varied at 4.5–9.5 hr, depending on its sensitivity. cAMP from the same samples was measured using a commercially available enzyme immunoassay kit and was found to be very similar to the enzymatic fluorometric assay. We conclude that this new assay is sensitive, safe, versatile, and inexpensive and can be used to measure cAMP in multiple types of tissue, including biopsy samples weighing <200 μg.

Key words: fluorescence, immunoassay, adenosine 3′:5′-monophosphate, enzymatic cycling, heart, liver, kidney

INTRODUCTION

Measurement of cAMP is generally performed using either radioimmunoassay (RIA), enzyme immunoassay (EIA), or high-performance liquid chromatography (HPLC). While these methods are sensitive, measurement of this fundamental second messenger is often expensive, lacks versatility, and may use radioactive substrate that is potentially harmful to both the operator and to the environment (1–3).

Given the biological importance of cAMP and the limited utility often associated with the currently available assay techniques, we developed a highly sensitive, nonradioactive, non-antibody-linked enzymatic fluorometric assay to simplify the measurement of cAMP. Essential to this new method is the initial enzymatic destruction of interfering compounds. The diester linkage is then cleaved, and newly generated AMP is measured enzymatically. The reactions are summarized below:

Step 1 Destruction of interfering compounds:

$$ATP \xrightarrow{\text{apyrase}} AMP + 2\,P_i$$
$$AMP + H_2O \xrightarrow{\text{5′ nucleotidase}} \text{adenosine} + P_i$$
$$\text{Adenosine} + H_2O \xrightarrow{\text{adenosine deaminase}} \text{inosine} + NH_3$$
$$\text{Glucose-6-phosphate} \xrightarrow{\text{alkaline phosphatase}} \text{glucose} + P_i$$

Step 2 Conversion of cAMP to AMP:

$$\text{cAMP} \xrightarrow{\text{phosphodiesterase}} \text{AMP}$$

Step 3 Conversion of AMP to ATP:

$$\text{AMP} + \text{ATP(trace)} \xrightarrow{\text{myokinase}} 2\,\text{ADP}$$
$$\text{ADP} + \text{phosphoenolpyruvate} \xrightarrow{\text{pyruvate kinase}} \text{ATP} + \text{pyruvate}$$

Step 4 Amplification by ATP–ADP cycling reaction:

$$\text{ATP} + \text{fructose} \xrightarrow{\text{hexokinase}} \text{fructose-6-phosphate} + \text{ADP}$$
$$\text{ADP} + \text{phosphoenolpyruvate} \xrightarrow{\text{pyruvate kinase}} \text{ATP} + \text{pyruvate}$$

Step 5 Fluorometric measurement of NADPH:

$$\text{Fructose-6-phosphate} \xrightarrow{\text{phosphoglucoisomerase}} \text{glucose-6-phosphate}$$
$$\text{Glucose-6-phosphate} + NADP^+ \xrightarrow{\text{glucose-6-phosphate dehydrogenase}} \text{6-phosphogluconolactone} + NADPH + H^+$$

Using this series of reactions, cAMP was measured in the extracts of heart, liver, and kidney of rat, before and after β-adrenergic stimulation. The results were confirmed using a commercially available EIA kit (Amersham).

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MATERIALS AND METHODS

Preparation of Tissue Extract

Eight male Sprague Dawley rats weighing 267.4 ± 3.0 g were anesthetized with pentobarbital (65 mg/kg, i.p.) and randomized into control (n=4) and stimulated groups (n=4). Electrocardiograms (ECGs) were obtained 15 min later for baseline values. At that time, control rats received no additional drug, while stimulated rats received aminophylline (20 mg/kg, s.q.), followed 5 min later by isoproterenol (16 μg/kg, s.q.). ECGs were repeated 15 min after administration of aminophylline, or no drug, in the case of the controls. Heart, liver, and kidney were rapidly excised and frozen in Cytocool (Stephens Scientific) precooled on crushed dry ice, 20 min after injection of aminophylline. Tissue samples trimmed approximately 500 mg were pulverized with a mortar and pestle on dry ice, added to 3 vol of precooled 0.5 N perchloric acid at 4°C and immediately homogenized on ice (4). Homogenization was performed using a Polytron (Kinematica GmbH LITTAU, Switzerland) with three sequential 15-sec bursts separated by 15 sec at a setting of 6. The homogenates were centrifuged at 2,000*g* for 30 min at 4°C. The supernatant solution was neutralized with 2 N KOH, frozen at –40°C, and thawed at 0°C to favor precipitation of the perchlorate. The neutralized supernatant was again centrifuged at 2,000*g* for 30 min at 4°C. The final supernatant was removed and stored at –80°C until cAMP content was measured. For the cAMP standard, a known amount of cAMP was solved in neutralized supernatant made from 0.5 N perchloric acid and 2 N KOH.

Enzymatic Fluorometric Measurement of cAMP

A volume of 3 μl of neutralized tissue extract or 3 μl of cAMP standard was added into a 10×75-mm Pyrex tube (Corning Laboratory Science Co.). In step 1, a volume of 25 μl of "cleaning" reaction mix (100 mM Tris–HCl, pH 8.0; 2 mM $MgCl_2$; 2 U/ml apyrase, EC 3.6.1.5; 2.5 U/ml 5′ nucleotidase, EC 3.1.3.5; 50 μg/ml adenosine deaminase, EC 3.5.4.4; 20 U/ml alkaline phosphatase, EC 3.1.3.1) was added to each reaction tube. The mixture was incubated for 30 min at 37°C. The enzymes were then destroyed by heating for 5 min at 95°C. A duplicate set of samples was similarly assayed and used as a tissue blank control (see below).

In step 2, a volume of 25 μl of a phosphodiesterase-containing reaction mix (100 mM Tris–HCl, pH 8.0; 2 mM $MgCl_2$; 0.01% bovine serum albumin (BSA); 40 μg/ml phosphodiesterase, EC 3.1.4.17) was added to the reaction products from step 1. After 30 min at 37°C, the reactions were terminated by heating at 95°C for 5 min. A tissue blank was generated by adding deactivated phosphodiesterase-containing reaction mix, which was preheated at 95°C for 5 min, to the parallel set of control samples from step 1.

In step 3, a volume of 25 μl of myokinase reaction mix (100 mM Tris–HCl, pH 8.0; 2 mM $MgCl_2$; 225 mM KCl; 20 nM ATP; 8 mM phosphoenolpyruvate; 2 mM dithiothreitol; 0.01% BSA; 24 μg/ml myokinase, EC 2.7.4.3; 50 μg/ml pyruvate kinase, EC 2.7.1.40) was added to the reaction products from step 2. The reaction was incubated for at least 5 hr at 37°C, or conveniently overnight at room temperature (20°C).

In step 4, a volume of 25 μl of "cycling" reaction mix (100 mM Tris–HCl, pH 8.0; 2 mM $MgCl_2$; 8 mM fructose; 0.01% BSA; 350 μg/ml pyruvate kinase; 400 μg/ml hexokinase, EC 2.7.1.1) was added to the reaction products from step 3 on ice. After 1 hr at 37°C, the reactions were terminated by heating at 90°C for 5 min. For assays requiring more sensitivity (<20 fmol cAMP detection/tube), the amount of cycling time for step 4 was increased to 3 hr or more.

In step 5, a volume of 800 μl of "indicator" reaction mix (50 mM Tris–HCl, pH 8.0; 200 μM $NADP^+$; 1 μg/ml phosphoglucoisomerase, EC 5.3.1.9; 1 μg/ml glucose-6-phosphate dehydrogenase, EC 1.1.1.49) was added to the reaction products from step 4. After 10 min at room temperature, the final concentration of NADPH was measured using a fluorometer (Optical Technology Devices, Inc.) at the setting in which a reading of 10 fluorometric units was equivalent to 1 nmol of NADPH in 900 μl of buffer (50 mM Tris–HCl, pH 8.0).

Individual reactions steps were verified by concurrently assaying internal standards using known concentrations of the appropriate substrate. For example, ATP and glucose-6-phosphate were used to assess step 1, while cAMP, AMP ATP, and fructose-6-phosphate were used to assess step 2, step 3, step 4, and step 5, respectively.

EIA for Measurement of cAMP

To confirm the reliability of new assay technique, cAMP in the same tissue extracts was measured using commercially available EIA kit for cAMP (Amersham). All samples were processed using the acetylated method.

Biochemicals and Statistics

Phosphoenolpyruvate, apyrase, 5′ nucleotidase, and fructose were obtained from Sigma Chemical Company. All other enzymes and substrates were obtained from the Boehringer-Mannheim Company. All assays were performed in triplicate and the data in the text are presented as mean ± SEM. The statistical comparisons of mean values were evaluated by Student's *t*-test. *P* values of <0.05 were considered significant.

RESULTS

"Cleaning" Reactions

The concentration of interfering compounds in tissue samples, such as ATP, ADP, AMP, and glucose-6-phosphate, is usually several orders of magnitude higher than that of cAMP. To solve this problem, we developed a series of enzy-

matic "cleaning" reactions that could destroy these compounds. A representative experiment demonstrating the effectiveness of the "cleaning" reactions using different concentrations of ATP is shown in Figure 1. There was no demonstrable increase in fluorescence in the presence of the "cleaning" reaction enzymes, and the slope of the resultant standard curve was 0. In contrast, an ATP standard curve without "cleaning" reaction enzymes in step 1 yielded a straight line with a slope of 22.7. Taken together, these results indicate that the "cleaning" reaction step removed all adenine nucleotides that might affect cAMP measurement. ATP as well as glucose-6-phosphate, tested in concentrations as high as 4 nmol/assay tube (data not shown), was no longer detectable after the "cleaning" reaction step.

Conversion of cAMP to AMP

Cleavage of the diester linkage of cAMP in step 2 is complete within 30 min at 37°C using phosphodiesterase in a concentration of 20 μg/ml. We tested a range of phosphodiesterase concentrations (10–120 μg/ml) and observed that small amounts of 5′ nucleotidase contamination in the phosphodiesterase resulted in a loss of AMP if the reaction times were more prolonged or the concentration of phosphodiesterase was greater than 20 μg/ml.

Specificity of the Assay

A representative cAMP standard curve (closed circles) is shown in Figure 2. The standard curve was linear and had a slope of 17.5 and fluorescence readings of 18.0–57.3 over a cAMP concentration range of 0–2.24 pmol. When the reaction in step 2 was carried out without phosphodiesterase, the standard curve (closed triangles) was linear with a slope of 0 and had a fluorescence reading of 17.0. The results indicate that the difference of fluorescence readings between the standard curves with and without phosphodiesterase specifically depends on added cAMP. An alternative method to demonstrate the specificity of this new assay is to include phosphodiesterase in the reaction mix in both step 1 as well as in step 2. The standard curve (not shown) was linear with a slope of 0 and had a fluorescence reading of 17.0 over a cAMP concentration range of 0–2.24 pmol, identical to that without phosphodiesterase in step 2 (closed triangles). The result indicates that different amount of cAMP was completely converted to AMP by phosphodiesterase and was also completely broken by "cleaning" reaction enzymes.

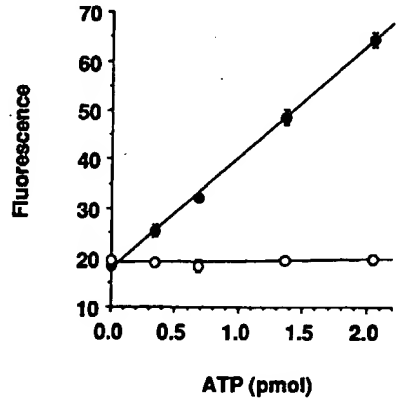

Fig. 1. Effectiveness of the "cleaning" reaction on different concentrations of ATP. The "cleaning" reaction step removed all adenine nucleotides. The ATP standard was incubated for 30 min at 37°C with either buffer (100 mM Tris–HCl, pH 8.0; 2 mM $MgCl_2$) (closed circles), or "cleaning" reaction mix (100 mM Tris–HCl, pH 8.0; 2 mM $MgCl_2$; 2 U/ml apyrase; 2.5 U/ml 5′ nucleotidase; 50 μg/ml adenosine deaminase; 20 U/ml alkaline phosphatase) (open circles) in step 1. After step 1, the assays were performed exactly as described in steps 2–5 in the Methods. Ten fluorescence units were equivalent to 1 nmol/tube of NADPH. Data expressed as mean ±SEM.

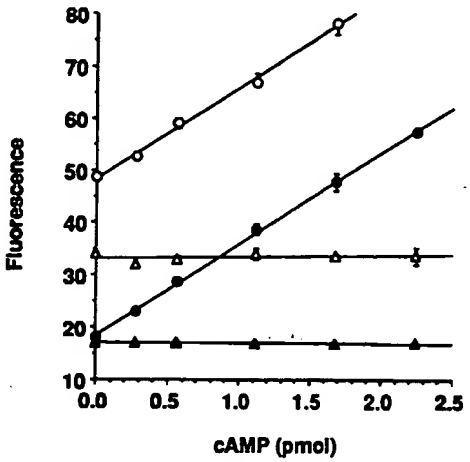

Fig. 2. Representative cAMP standard curves without (closed circles and triangles) and with 3 μl of rat liver extract stimulated by β-adrenergic agonists (open circles and triangles). These cAMP standards were incubated for 30 min with either a phosphodiesterase-containing mix (25 mM Tris–HCl, pH 8.0; 2 mM $MgCl_2$; 0.02% bovine serum albumin; 40 μg/ml phosphodiesterase) (open and closed circles) or a deactivated phosphodiesterase-containing reaction mix, preheated at 95°C for 5 min (open and closed triangles) in step 2 (see Methods). Ten fluorescence units are equivalent to 1 nmol/tube of NADPH. Data are shown as mean ±SEM.

Sensitivity of the Assay

The sensitivity of the cAMP assay is dependent on a number of factors, such as the amount of ATP added in step 3, and the cycling duration, reaction volume, and enzyme concentration in step 4. The initial concentration of ATP in the conversion of AMP to ADP by myokinase in step 3 is most critical to the assay sensitivity. Low concentration of ATP decreases the cAMP standard blank but requires a longer duration for the step 3 reaction for completion. We used 0.5 pmol of ATP/assay tube to maintain assay sensitivity and complete the reaction within 5 hr. We found it convenient to set up the assay in the afternoon and allow step 3 to proceed overnight.

To increase assay sensitivity, we increased the cycling time in step 4 from 1 hr to 2 or 3 hr. As shown in Figure 3, increasing the duration of step 4 resulted in greater assay sensitivity enough t detect differences as small as 21 fmol of cAMP between different samples. On the other hand, higher concentration of initial ATP in step 3 resulted in a higher cAMP standard blank and decreased assay sensitivity, but it could reduce the overall reaction time in step 3 to less than 2 hr. When assaying cAMP in the 4–40-pmol range, 2 pmol ATP/reaction tube in step 3, and a 10-fold lower concentration of hexokinase and pyruvate kinase in reaction step 4 can be used. In this fashion, the overall assay can be conveniently performed in less than 5 hr.

Effects of Tissue Extract on the Fluorometric Assay

The effects of tissue extract on the new assay were assessed. A representative cAMP standard curve with 3 µl of rat liver extract (open circles) is shown in Figure 2. The standard curve was linear and had a slope of 17.6 and fluorescence readings of 48.5–78.3 over a cAMP concentration range of 0–1.68 pmol. The similarity of the slopes of the cAMP standard curves, in the presence (open circles) and absence (closed circles) of liver extract, provides proof that the tissue extract does not affect the kinetics of this new assay. The similar parallel shift of standard curve was also observed with the addition of extract of heart or kidney (data not shown).

Intrinsic Fluorescence Activity of Tissue Extract

It is known that tissue extracts prepared as described above may have intrinsic fluorescence activity and contribute to the total fluorescence readings (4). When the reaction in step 2 was carried out without phosphodiesterase, the standard curve with 3 µl of stimulated liver extract (open triangles) was linear with a slope of 0 and had a fluorescence reading of 33.1, parallel to that without tissue extract (closed triangles), as shown in Figure 2. As cAMP cannot effect the total fluorescence reading without phosphodiesterase, as described above, the difference of fluorescence readings in these standard curves (open and closed triangles) most likely originates from the intrinsic tissue fluorescence activity. In this example, 15.1 fluorescence units (33.1 minus 18.0) were due to intrinsic tissue fluorescence, while 15.1 fluorescence units (48.2 minus 33.1) were due to tissue cAMP. In the present assay, the intrinsic fluorescence of heart, liver, and kidney extract (3 µl) in a total volume of 900 µl of buffer (50 mM Tris–HCl, pH 8.0) was 7.7 ± 0.6, 10.2 ± 1.1, and 12.1 ± 0.9 fluorescence units in the control group, and 7.4 ± 0.3, 11.4 ± 1.3, and 15.0 ± 1.3 fluorescence units in the stimulated group, respectively. The amount of intrinsic tissue extract fluorescence varied depending on the tissue type, but there were no significant differences between the control and stimulated groups in each organ.

To provide further proof that the increased fluorescence observed after the addition of tissue originated from a combination of intrinsic cAMP content and intrinsic tissue fluorescence, and not from incomplete degradation of contaminant noncyclic adenine nucleotides in the "cleaning" reactions, up to 2 nmol of exogenous ATP was added to some tissue extracts. In the presence of "cleaning" reaction enzymes, there was no incremental increase in fluorescence in the ATP-supplemented extracts.

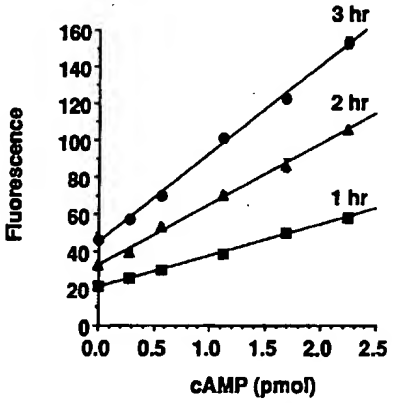

Fig. 3. Demonstration of the effects of increasing duration of the cycling reactions in step 4 on cAMP standards. With a reaction time of 1 hr (closed squares), 2 hr (closed triangles), and 3 hr (closed circles) in step 4, the assay sensitivity increases proportionally. The equations for the cAMP standard curves with 1, 2, and 3 hr of cycling are $y=16.8x+20.9$, $y=32.7x+32.5$, and $y=47.7x+44.9$, respectively. Ten fluorescence units are equivalent to 1 nmol/tube of NADPH. Data are shown as mean ±SEM.

Calculation of Tissue cAMP Content

The effect of intrinsic tissue fluorescence can be minimized by using enzymatic cycling, since intrinsic tissue fluorescence is not amplified (4). As the cycling time is increased, the intrinsic tissue fluorescence contribution to the total measured fluorescence decreases. However, with the use of tissue blank values to calculate the amount of cAMP in "unknown" samples, the potential problem of intrinsic fluorescence is completely eliminated as the amount of cAMP is calculated by the difference between fluorometric tissue reading and tissue blank reading. For this reason, a tissue blank was used in all experiments to determine the amount of cAMP. The amount of the cAMP in the unknown sample was calculated using the following formula:

$$\frac{\text{Tissue fluorescence reading–tissue blank fluorescence reading}}{\text{slope of standard curve}}$$

Application of New Assay to Tissue Extracts of Rat

To demonstrate the use and reliability of this new assay, we measured cAMP in a number of different rat organs. cAMP was measured in anesthetized rats in the absence or

presence of β-adrenergic stimulation. The basal heart rate was 379 ± 10 beats/min in the control group and 334 ± 20 beats/min in the stimulated group (P=NS). The heart rate of stimulated rats increased to 453±10 beats/min after β-adrenergic agonist stimulation and was significantly higher than baseline values (P<0.01). The cAMP content in heart, liver, and kidney was measured using the new method and commercially available EIA kit. As shown in Figure 4, the results were nearly identical. This value was normalized to tissue wet weight but could be normalized to other tissue properties, such as protein or DNA content. The levels of cAMP content of heart, liver, and kidney in the stimulated group were all significantly higher than those in the control group. There was a 2.3-, 2.0-, and 1.7-fold increase in cAMP content after β-adrenergic stimulation in heart, liver, and kidney, respectively. We routinely assayed sets of 3–6 tissue extracts/unknown sample. Results of multiple analyses of a single sample did not differ by more than 10%, with variability usually less than 5%.

DISCUSSION

Given the expense, time, radioactivity, and limited flexibility associated with the currently available assays for cAMP, including RIA, EIA or HPLC, we developed a highly sensitive nonradioactive enzymatic fluorometric assay. Assay sensitivity can be easily varied, depending on the requirements of a particular sample. This method can be used to detect <60 fmol cAMP/assay with a 1-hr cycling time in step 4. With a 3-hr cycling time in step 4, detection is increased to <21 fmol cAMP/assay tube. The absolute values of tissue cAMP content, as well as the fold stimulation obtained from both enzymatic fluorometric assay and EIA method, were nearly identical, and the results were similar t those previously reported using different techniques (5). However, the enzymatic fluorometric approach may offer a number of potential advantages over current methods, including (1) expenses on a cost/assay basis are about 90% less than those associated with a commercially available EIA or RIA kit; (2) the price of equipment for enzymatic fluorometric assay is far less than that of HPLC; (3) risks and costs associated with radioactivity are eliminated; and (4) the standard curve of enzymatic fluorometric assay is linear over a wide range of cAMP (21–40,000 fmol), while that of EIA or RIA is parabolic and requires careful dilution of samples to get the optimal concentration for assay (25–1,600 fmol).

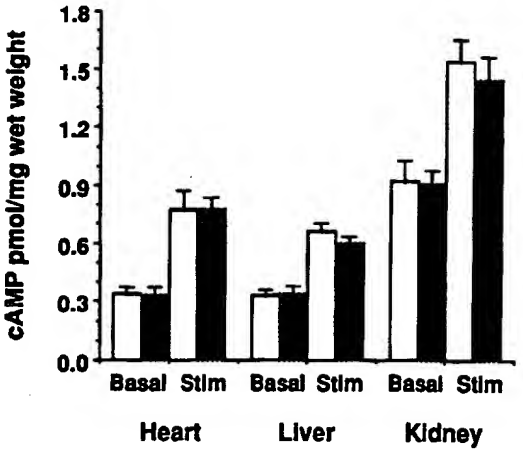

Fig. 4. Concentration of cAMP (pmol/mg wet weight) of rat heart, liver, and kidney under basal conditions (Basal) and after 20 min of stimulation with aminophylline (20 mg/kg, s.q.) and 15 min of stimulation with isoproterenol (16 μg/kg, s.q.) (Stim). cAMP was measured using an enzymatic fluorometric assay (open rectangles) and a commercially available enzyme-immunoassay (closed rectangles). Absolute values as well as the fold stimulation from both method were nearly identical. Data are expressed as mean ±SEM.

Breckenridge (6) first described the conception of the enzymatic fluorometric cAMP assay for brain in 1964. Despite modifications of this original description, including the addition of a cellulose thin-layer chromatography step to increase sensitivity and specificity, application of enzymatic fluorometric assays for measurement of cAMP to other organs has been limited secondary to extremely low tissue levels of cAMP and interfering compounds that can be several thousand times higher in concentration than cAMP (7). Thus, the most important change in the current assay compared with assays described by Breckenridge (6) is the addition of adenosine deaminase and 5′ nucleotidase to the "cleaning" reaction step. When our "cleaning" reaction was used, there was effectively no difference between the tissue blank reading and the intrinsic tissue fluorescence. In contrast, with the use of the method described by Breckenridge (6), the tissue blank reading was 6.5 times greater than the intrinsic tissue fluorescence, which was greater than the standard curve range for that experiment (0–2.24 pmol). The results suggest that by generating NH_3 with adenosine deaminase, the adenine nucleotide dephosphorylation reactions are driven to completion.

We recently described a similar fluorometric approach for the measurement of adenylate cyclase (8). In those studies, newly synthesized cAMP was measured after a different series of "cleaning" reactions with amplification reactions that use the known stimulatory effects of AMP on glycogen phosphorylase *a*. In the current study, however, we selected the ATP–ADP cycling reaction to amplify the ATP signals, as it is reliable and more sensitive, when comparable reaction volumes are used and it does not rely on the specific activity of glycogen phosphorylase *a*, which can be somewhat variable from lot to lot, at least in the currently commercially available forms (Sigma Chemical Co.). Furthermore, substitution of fructose for glucose in the ATP–ADP cycling reactions in step 4 yielded twice as much NADPH after 1 hr of cycling under the same experimental conditions. Other alternative amplification of ATP are also feasible. For example, a luminometric method can be used to amplify the ATP in the reaction products of step 3 (5).

In this study, we observed significant intrinsic tissue extract fluorescence. This was most likely due, in part, to the extract method and tissue preparation. Although standard methods were used (4), we did not observe this degree of intrinsic tissue fluorescence in the measurement of cardiac cells in culture (9). Nonetheless, the possibility that intrinsic tissue fluorescence may contribute to the overall fluorescence reading should be assessed with each tissue extraction. In this study, the potential problem of intrinsic fluorescence is completely eliminated with the use of tissue blank values to calculate the cAMP content, since the amount of cAMP is calculated by the difference between the fluorometric tissue reading and tissue blank reading. The enzymatic fluorometric measurement of cAMP in the same extract was essentially identical to that with the EIA method, and the results were similar to those reported by others using the same type of tissue (5).

In summary, this nonradioactive fluorometric assay offers an alternative to current techniques which are often more costly, less versatile and potentially harmful to the operator and the environment. The examples provided in this report demonstrate the potential widespread application of this new technique to measure cAMP in multiple types of tissue, including biopsy samples weighing less than 0.2 mg.

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NRC

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AN 1999:271208 BIOSIS
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TI Measurement of adenylate cyclase activity in the minute bovine ciliary epithelial cells during the pharmacological stimulation of adrenergic and cholinergic receptors.
AU Chiba, T. (1); Kashiwagi, K. (1); Sugiyama, A. (1); Hashimoto, K. (1); Tsukahara, S. (1)
CS (1) Yamanashi Medical University, Yamanashi Japan
SO IOVS, (March 15, 1999) Vol. 40, No. 4, pp. S496.
Meeting Info.: Annual Meeting of the Association for Research in Vision and Ophthalmology Fort Lauderdale, Florida, USA May 9-14, 1999 Association for Research in Vision and Opthalmology

2614—B489

NITRIC OXIDE PRODUCTION FROM ISOLATED PORCINE AND HUMAN CILIARY PROCESSES . T. Haufschild[1], M. R. Tschudi[2], J. Flammer[1], T. F. Luescher[2], I. O. Haefliger[1]. Laboratory of Pharmacology and Physiology, University Eye Clinic, Basel Switzerland[1], Cardiovascular Research, Cardiology, University Hospitals, Zurich, Switzerland[2].

Purpose: Nitric Oxide (NO) is a bioregulatory molecule involved in many physiological and pathophysiological processes in the eye. This study addresses the direct release of NO from porcine and human ciliary processes in vitro.
Methods: Porcine ciliary processes and iris were dissected free and either used fresh or thawed after storage at -70°C. Human ciliary processes were taken at the time of autopsy and frozen until thawed for measurements. The NO production was measured with a porphyrinic microsensor placed on the surface of the specimen in a HBSS-buffer at 37°C.
Results: After 30 minutes (required for equilibration) the analytic signal reached a stable level in the porcine ciliary processes and a much lower one in the porcine iris and human ciliary processes. In porcine ciliary processes the signal was significantly decreased by the NO formation inhibitor NG-nitro-L-arginine methyl ester (L-NAME, 2 x 0.1 mM c. g. 1 mM, $n = 4 - 5$, $p < 0.001$), but not by the inactive analog NG-nitro-D-arginine methyl ester (D-NAME, 1 mM, $n = 4$). In human ciliary processes the signal was also significantly decreased by L-NAME (2 x 0.1 mM, $n = 5$, $p < 0.001$).
Conclusions: Nitric oxide - measured with a porphyrinic microsensor - is produced from isolated porcine and human ciliary processes through the activity of a nitric oxide synthase.
CR: None Support: Swiss National Science Foundation Grant # 32-52783.97 (Bern, Switzerland), the Schwickert Foundation (Basel, Switzerland), and the Velux Foundation (Zurich, Switzerland).

2615—B490

NITRIC OXIDE - cGMP PATHWAY ACTIVATION INDUCES MEMBRANE POTENTIAL DEPOLARIZATION IN PORCINE CILIARY EPITHELIUM . J. C. Fleischhauer, J. Flammer, I. O. Haefliger, Laboratory of Ocular Pharmacology and Physiology, University of Basel.

Purpose: To investigate if in isolated porcine ciliary processes activation of the nitric oxide (NO) - guanylate cyclase (GC) - 3',5'-cyclic guanosine monophosphate (cGMP) pathway modulates ciliary epithelial transmembrane potential. Methods: Changes in transmembrane epithelial potential induced by the NO donor sodium-nitroprusside (SNP; 100 µM; $n = 8$) or the cGMP analog Rp-8-para-chloro-phenylthioguanosine-3',5'-cyclic monophosphate (Rp-8-pCPT-cGMP; 100 µM; $n = 8$) were measured with microelectrodes in the presence or in the absence of the GC inhibitor 1-H-(1,2,4)oxa-diazole-(4,3-a)quinoxalin-1-one (ODQ; 10 µM; $n = 5$). The effect of Rp-8-pCPT-cGMP was also assessed in the presence of the anionic channel inhibitors niflumic acid (100 µM; $n = 5$), diiso-thiocyanato-stilbene, 2,2' disulfonic acid (DIDS; 1 mM; $n = 5$), and anthracene-9-carboxylic acid (9-AC; 1 mM; $n = 5$). Results: Membrane depolarizations were induced by SNP (4.6 ± 1.1 mV depolarization; mean ± SEM) and Rp-8-pCPT-cGMP (10.6 ± 1.1 mV). Only depolarizations evoked by SNP but not by Rp-8-pCPT-cGMP were inhibited ($P < 0.001$) by ODQ. Depolarizations induced by Rp-8-pCPT-cGMP were inhibited ($P < 0.001$) by niflumic acid (1.3 ± 0.5 mV), DIDS (0.6 ± 0.5 mV), and by 9-AC (4.9 ± 0.7 mV). In quiescent preparations, DIDS (-4.9 ± 1.3 mV) and 9-AC (-5.5 ± 0.5 mV) evoked significant ($P < 0.001$) hyperpolarizations, but not niflumic acid (0.8 ± 0.4 mV) which evoked a small and non significant depolarization. Conclusions: In isolated porcine ciliary processes, activation of the NO - cGMP pathway induces an epithelial transmembrane depolarization by a process which appears to involve anion channels.
CR: None Support: Swiss National Science Foundation Grant #32-52783.97 (Bern, Switzerland) and the Roche Research Foundation (Basel, Switzerland).

2616—B491

NITRIC OXIDE SYNTHASE IMMUNOSTAINING DISTRIBUTION IN PORCINE CILIARY PROCESSES . I. O. Haefliger, C. Champion, J. Flammer, P. Meyer. University Eye Clinic, Basel, Switzerland.

Purpose: To investigate the distribution of nitric oxide synthase (NOS) isoforms immunostaining in isolated porcine ciliary processes.
Methods: Within 10 minutes after animals' death, specimen were dissected free and placed into liquid nitrogen. For light microscopy immunohistochemical procedures, 9-10 µm sections (cryostat, -25 degrees Celsius) were mounted on chrome alum-gelatin slides. After paraformaldehyde (PFA) 4 % fixation, sections were exposed to specific monoclonal antibodies against neuronal NOS (nNOS), macrophage NOS (macNOS), or endothelial NOS (ecNOS), and then immunostained with a FITC-conjugated secondary antibody.
Results: In porcine ciliary epithelium, immunostaining was marked for nNOS (between non-pigmented and pigmented cells) and mild for macNOS (non pigmented cells' cytoplasm). Vascular endothelial cells immunostained for ecNOS.
Conclusions: In porcine ciliary epithelium (where aqueous humor is produced) there is an intense nNOS immunostaining at the junction between non-pigmented and pigmented ciliary cells.
CR: None Support: Swiss National Science Foundation Grant #32-52783.97 (Bern, Switzerland), the Schwickert Foundation (Basel, Switzerland), and the Velux Foundation (Zurich, Switzerland).

2617—B492

MEASUREMENT OF ADENYLATE CYCLASE ACTIVITY IN THE MINUTE BOVINE CILIARY EPITHELIAL CELLS DURING THE PHARMACOLOGICAL STIMULATION OF ADRENERGIC AND CHOLINERGIC RECEPTORS T. Chiba, K. Kashiwagi, A. Sugiyama, K. Hashimoto, S. Tsukahara. Yamanashi Medical University.

Purpose: To measure adenylate cyclase activity using a newly developed highly-sensitive fluorometric assay technique in the bovine ciliary epithelial cells during the pharmacological stimulation of adrenergic and cholinergic receptors. Methods: Ten µl of membrane (30-50 µg/total protein) preparations from bovine ciliary epithelium were employed for the assay. Prior to be adopted the assay, membrane preparations were incubated with adrenergic or cholinergic reagents: norepinephrine (10^{-7} to 10^{-5} M), forskolin (10^{-5}M), and carbachol ($10^{-7}$to 10^{-5}M). After the initial enzymatic destruction of non-cyclic adenine nucleotides and phosphorylated metabolites, the diester linkage of cyclic AMP was cleaved and then converted to ATP. The ATP was enzymatically amplified to about 10,000 times of fructose-6-phosphate. The NADPH formed when the fructose-6-phosphate was converted to 6-phohphoguconolactone was measured fluorometrically. Results: Basal and forskolin-stimulated maximum adenylate cyclase activities (pmol/mg protein/min) were 29.6±7.6 and 86.6±7.2 (mean±SE), respectively. Norepinephrine increased the adenylate cyclase activity in a dose-dependent manner, while carbachol hardly affected the activity. Conclusions: These results indicate that the adenylate cyclase activity can be measured in the minute ciliary epithelium, moreover suggest that the current assay can be applied to assess the efficacy of ophthalmic solutions or systemic drugs on adenylate cyclase activity in the eye.
CR: None Support: Japanese Monbusyo No.09771416 .

2618—B493

POTENTIAL SITES AND MECHANISMS OF ACTION OF TNPA: A DA-2 DOPAMINE RECEPTOR AGONIST((T.-C Chu, D.E Potter)) Department of Pharmacology and Toxicology, Morehouse School of Medicine, Atlanta, GA

Purpose. This study examined potential sites of action for TNPA in ciliary processes and quantified effects of TNPA on IOP and norepinephrine release. Methods. Immunocytochemistry of the DA-2 dopamine receptor was performed in ciliary processes of normal and sympathetically denervated rabbits utilizing a goat polyclonal DA-2 dopamine receptor IgG and anti-goat IgG-FITC. IOP(mmHg) of rabbits was measured by pneumatonometry. Alterations of norepinephrine levels in aqueous humor were measured by HPLC-EC. Drugs utilized were R(-)-2,10,11-tri-hydroxy-N-propyl-noraporphine hydrobromide (TNPA), a DA-2 dopamine receptor agonist and raclopride, a DA-2 dopamine receptor antagonist. Results. Immunolocalization experiments demonstrated that DA-2 dopamine receptors are present in ciliary processes of normal rabbits but staining was minimal in that of sympathectomized rabbits. The levels of norepinephrine in aqueous humor of sympathectomized rabbits were 17% of the levels of normal rabbits. Moreover, the levels of norepinephrine in aqueous humor were reduced by 50% and 80% at 1, 2 hr, respectively, following topical application of TNPA (75µg). The decline in IOP induced by TNPA(75µg) was 4.5 and 8 mmHg at 1, 2 hr, respectively. Pretreatment with raclopride (750µg) antagonized the reduction of IOP and levels of norepinephrine evoked by a challenge with TNPA (75µg, 2 hr). Conclusions. Immunohistochemical identification of DA-2 dopamine receptors in ciliary processes coupled with the marked reduction in staining following denervation suggest that DA-2 dopamine receptors are on sympathetic nerves. Antagonism of TNPA-induced suppression of IOP and aqueous norepinephrine levels by raclopride corroborate that a primary site of action of TNPA is the sympathetic nerve.
CR:None Support: NIH grants EY06338 and S06GM08248-12.

2619—B494

STUDIES OF [^{3}H]-5-HYDROXYTRYPTAMINE RELEASE FROM BOVINE AND HUMAN ANTERIOR UVEA. ((S.O. Awe, S.E. Ohia,*N.A. Sharif, C.A. Opere and J Adams)) Creighton University Health Sciences Center, Omaha, NE 68178, *Alcon Laboratories, Inc., Fort Worth,TX 76134, USA.

Although serotonin (5-HT) receptors have been localized in tissues of the anterior uvea, their functional role is yet to be fully determined. Purpose. To characterize prejunctional 5-HT autoreceptors that regulate the release of [^{3}H]-5-HT from bovine and human iris-ciliary bodies. Methods. Isolated bovine and human tissues were incubated in oxygenated Krebs solution containing 1.6 µM [^{3}H]-5-HT, pargyline (10 µM) and flurbiprofen (3 µM). After incubation, tissues were prepared for superfusion studies. Release of [^{3}H]-5-HT was elicited by 300 d.c electrical pulses applied at 200 mins (S_1) and 232 mins (S_2) after onset of superfusion. Results. Electrical stimulus was effective in eliciting [^{3}H]-5-HT release from both bovine and human tissues yielding S_2/S_1 ratios of 0.96 ± 0.08 ($n = 6$)and 1.06 ± 0.11($n = 6$), respectively. Both tetrodotoxin (0.1 µM) and zero-calcium buffer caused a 69% and 65% inhibition of evoked [^{3}H]-5-HT release from bovine tissues, respectively. Exogenously applied 5-HT (10 nM - 1 µM) produced a concentration-related enhancement of evoked [^{3}H]-5-HT overflow without affecting basal tritium efflux in bovine and human tissues. Agonists selective for 5-HT receptor subtypes also enhanced field-stimulated [^{3}H]-5-HT overflow with the following rank order of potency in bovine tissues: m-chlorophenylbiguanide > 5-carboxamidotryptamine > 2-methyl-5HT = 5-methoxy-dimethyltryptamine > 5-HT >> L-694247 = α-methyl-5-HT > CGS-12066A. Human iris-ciliary bodies were less sensitive than bovine tissues to the excitatory effect of 5-HT and m-chlorophenylbiguanide on evoked [^{3}H]-5-HT release. Conclusions. In the anterior uvea, electrically-evoked release of [^{3}H]-5-HT is via a calcium-dependent process that is subject to regulation by excitatory prejunctional 5-HT autoreceptors. Agonists selective for 5-HT$_2$ receptors were the most potent in enhancing evoked [^{3}H]-5-HT from the bovine iris-ciliary body.
CR: C2, F*

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=> d his

     (FILE 'HOME' ENTERED AT 10:15:56 ON 22 MAY 2003)

     FILE 'CA' ENTERED AT 10:16:04 ON 22 MAY 2003

     FILE 'REGISTRY' ENTERED AT 10:16:12 ON 22 MAY 2003
L1             1 S APYRASE/CN
L2             3 S ADENOSINE DEAMINASE/CN
L3             1 S ALKALINE PHOSPHATASE/CN

     FILE 'CA' ENTERED AT 10:18:18 ON 22 MAY 2003
                S 9001-78-9/REG#

     FILE 'REGISTRY' ENTERED AT 10:18:38 ON 22 MAY 2003
L4             1 S 9001-78-9/RN

     FILE 'CA' ENTERED AT 10:18:38 ON 22 MAY 2003
L5         31944 S L4
                S 9000-95-7/REG#

     FILE 'REGISTRY' ENTERED AT 10:18:51 ON 22 MAY 2003
L6             1 S 9000-95-7/RN

     FILE 'CA' ENTERED AT 10:18:51 ON 22 MAY 2003
L7           706 S L6
L8            28 S L5 AND L7
                S S  9026-93-1/REG#

     FILE 'REGISTRY' ENTERED AT 10:22:09 ON 22 MAY 2003
L9             1 S 9026-93-1/RN

     FILE 'CA' ENTERED AT 10:22:09 ON 22 MAY 2003
L10         3793 S L9
L11            6 S L10 AND L8

     FILE 'WPIDS' ENTERED AT 10:23:29 ON 22 MAY 2003
L12           38 S APYRASE OR DIPHOSPHOHYDROLASE
L13          328 S ADENOSINE DEAMINASE
L14            3 S L12 AND L13
L15            2 S ALKALINE PHOPHATASE
L16         1477 S ALKALINE PHOSPHATASE
L17            0 S ALKALINE PHOSPHOHYDROLASE
L18         1477 S L16 OR L17
L19            2 S L14 AND L18

     FILE 'USPATFULL' ENTERED AT 10:26:20 ON 22 MAY 2003
L20         1383 S 9001-78-9/RN
L21           38 S 9000-95-7/RN
L22            0 S 9026-93-1
L23          239 S APYRASE OR DIPHOSPHOHYDROLASE
L24          241 S L23 OR L21
L25         2754 S ADENOSINE DEAMINASE
L26           18 S ADENOSINE AMINOHYDROLASE
L27           18 S L25 AND L26
L28        25511 S ALKALINE PHOSPHATASE
L29            1 S ALKALINE PHOSPHOHYDROLASE
L30        25598 S L29 OR L28 OR L20
L31            5 S L30 AND L27

=> log hold
COST IN U.S. DOLLARS                          SINCE FILE      TOTAL
                                                 ENTRY    SESSION
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FULL ESTIMATED COST	17.69	116.61

DISCOUNT AMOUNTS (FOR QUALIFYING ACCOUNTS)	SINCE FILE ENTRY	TOTAL SESSION
CA SUBSCRIBER PRICE	0.00	-6.82

SESSION WILL BE HELD FOR 60 MINUTES
STN INTERNATIONAL SESSION SUSPENDED AT 10:30:36 ON 22 MAY 2003

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=> d his

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     FILE 'CA' ENTERED AT 12:54:14 ON 22 MAY 2003
L1         22105 S PHOSPHODIESTERASE
L2         75594 S EDTA
L3           369 S L1 AND L2
L4         82068 S INACTIVATION
L5            20 S L3 AND L4
L6          3298 S CAMP PHOSPHODIESTERASE
L7         12145 S CHELATOR
L8            17 S L6 AND L7

     FILE 'REGISTRY' ENTERED AT 13:03:34 ON 22 MAY 2003
L9             1 S 9036-21-9/RN

     FILE 'LCA' ENTERED AT 13:04:10 ON 22 MAY 2003

     FILE 'CA' ENTERED AT 13:08:16 ON 22 MAY 2003
                S 9036-21-9/REG#

     FILE 'REGISTRY' ENTERED AT 13:08:27 ON 22 MAY 2003
L10            1 S 9036-21-9/RN

     FILE 'CA' ENTERED AT 13:08:27 ON 22 MAY 2003
L11         4892 S L10
L12        85619 S L2 OR L7
L13           65 S L11 AND L12

     FILE 'BIOSIS' ENTERED AT 14:01:06 ON 22 MAY 2003
L14            3 S (MINUTE BOVINE CILIARY)/TI

=> log hold
COST IN U.S. DOLLARS                                SINCE FILE      TOTAL
                                                       ENTRY    SESSION
FULL ESTIMATED COST                                     9.87      99.98

DISCOUNT AMOUNTS (FOR QUALIFYING ACCOUNTS)          SINCE FILE      TOTAL
                                                       ENTRY    SESSION
CA SUBSCRIBER PRICE                                     0.00      -7.44

 SESSION WILL BE HELD FOR 60 MINUTES
STN INTERNATIONAL SESSION SUSPENDED AT 14:04:25 ON 22 MAY 2003
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